Available online at www.sciencedirect.com

SCIENCE DIRECT®

Virology 324 (2004) 400–411

VIROLOGY

www.elsevier.com/locate/yviro

Tryptophan scanning mutagenesis of aromatic residues within the polymerase domain of HIV-1 reverse transcriptase: critical role of Phe-130 for p51 function and second-site revertant restoring viral replication capacity

Isabel Olivares,[a] Mónica Gutiérrez-Rivas,[a,b,1] Cecilio López-Galíndez,[a] and Luis Menéndez-Arias[b,*]

[a] *Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain*

[b] *Centro de Biología Molecular "Severo Ochoa", Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain*

Received 12 January 2004; returned to author for revision 10 February 2004; accepted 8 April 2004

Available online 18 May 2004

Abstract

The effects on virus viability and reverse transcriptase (RT) function of substituting Trp for Tyr or Phe residues within the polymerase domain of human immunodeficiency virus type 1 (HIV-1) RT have been analyzed with an infectious HIV-1 clone. Viruses containing mutations Y56W, F61W, F87W, F116W, Y127W, Y144W, F171W, Y181W, Y183W, Y188W, F227W, or Y232W in their RT-coding regions were viable and showed replication capacities similar or slightly reduced in comparison with the wild-type HIV-1. However, RTs bearing mutations F77W or Y146W had a dNTP-binding defect, rendering nonviable viruses. HIV-1 carrying RT mutations F124W or F130W replicated very poorly, but compensatory changes (K83R for F124W, and T58S for F130W) were selected upon passaging the virus in cell culture. The amino acid substitution F130W diminishes the stability of the 51-kDa subunit of the RT (p51) and impairs polyprotein processing in virus-infected cells, an effect that can be mitigated when T58S is found in p51.

Keywords: HIV; Reverse transcriptase; Fitness; Polymerase; Drug resistance; Retrovirus; Mutagenesis

Introduction

The human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) catalyzes the synthesis of proviral DNA using the viral RNA as template (for a review, see Telesnitsky and Goff, 1997). It is a multifunctional enzyme, possessing RNA- and DNA-dependent DNA polymerase, RNase H, strand transfer, and strand-displacement activities. The process of reverse transcription is error prone and contributes to the high genetic variability of HIV-1 (for recent reviews, see Menéndez-Arias, 2002a; Svarovskaia et al., 2003). HIV-1 RT is a heterodimer composed of subunits of 66 kDa (p66) and 51 kDa (p51). Subdomains within each subunit are termed "fingers", "palm", "thumb", and "connection" based on X-ray crystal structure and standard subdomain classification in various polymerases (Kohlstaedt et al., 1992; Joyce and Steitz, 1995). The heterodimer is generated after the viral protease cleaves one of the RNase H domains of an asymmetric p66/p66 homodimer (Di Marzo Veronese et al., 1986). The DNA-binding cleft and the polymerase active-site residues are exposed in the "open" conformation of p66. On the other hand, the 51-kDa subunit is closely folded, with its catalytic residues occupying an internal position in the molecule (Ding et al., 1998; Huang et al., 1998; Kohlstaedt et al., 1992). In addition to its structural role, p51 has been implicated in loading the p66 subunit on the template-primer (Harris et al., 1998) and in maintaining the proper conformation of p66 during initiation of reverse transcription (Arts et al., 1996).

* Corresponding author. Fax: +34-914974799.
E-mail address: lmenendez@cbm.uam.es (L. Menéndez-Arias).
[1] Present address: CISA-INIA, Valdeolmos, Madrid, Spain.

0042-6822/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2004.04.010

Amino acid sequence alignments of the polymerase domain of retroviral RTs have revealed a series of homology regions that are conserved in RT-related DNA polymerases as well as in RNA-dependent RNA polymerases (Nakamura et al., 1997; O'Reilly and Kao, 1998; Xiong and Eickbush, 1990). Conserved regions include motifs 1 and 2 in the fingers subdomain, which contain residues such as Lys-65 and Arg-72 that participate in triphosphate binding, and motifs A, B′, C, D, and E that contain residues within the fingers and palm subdomains of the RT. The catalytic residues are found in motifs A (Asp-110) and C (Asp-185 and Asp-186). Motif B′ participates in dNTP binding through Gln-151, which is part of the highly conserved sequence Leu-Pro-Gln-Gly, and motif E is part of the "primer grip" formed by residues involved in maintaining the primer terminus in an appropriate orientation for nucleophilic attack of an incoming dNTP (Ding et al., 1998; Huang et al., 1998).

Aromatic residues such as Tyr-115 and Phe-160 within motifs A and B′, respectively, are important to maintain the integrity of the dNTP binding site, and nonconservative substitutions at these positions lead to the loss of RT function and virus viability (Cases-González et al., 2000; Gutiérrez-Rivas et al., 1999; Martín-Hernández et al., 1996; Olivares et al., 1999). Previously, we showed that the substitution of Trp for Tyr-115 impaired DNA polymerase activity and rendered a virus that replicated very poorly in MT-4 cells. However, in transfection experiments carried out with proviral DNA having the RT mutation Y115W, we found a compensatory substitution in motif E (M230I) that restored the replication capacity of the virus (Olivares et al., 1999). The emergence of a second-site revertant was favored by the requirement of two simultaneous nucleotide substitutions to convert the Trp codon (UGG) into a Tyr codon (UAU, UAC). In this work, we have extended our analysis to the other Tyr and Phe residues located within positions 40 and 240 of the RT polymerase domain, which include the conserved motifs 1, 2, A, B′, C, D, and E. Sixteen mutant proviruses containing Phe→Trp or Tyr→Trp substitutions were obtained and assayed for viability, and RTs from viruses showing diminished replication capacity were purified and characterized. A novel compensatory mutation in p51 was identified after passaging in cell culture a replication-deficient virus carrying the mutation F130W.

Results

In vitro replication of HIV-1 mutants

The effects on virus replication of substituting Trp for Tyr or Phe at different positions of the RT polymerase domain were assessed after introducing the corresponding mutations into the proviral genome. COS-1 cells were transfected with WT (HIV-1 subtype B strain 89ES061) or mutated proviral DNA, and after addition of MT-4 cells, co-culture supernatants were monitored at various times for RT activity. Maximum levels of RT activity were detected 12 days after transfection with WT DNA (Table 1), and similar results were obtained with mutants Y56W, F87W, F116W, Y127W, Y144W, F171W, Y181W, Y183W, Y188W, F227W, and Y232W that showed RT activity peaks between days 10 and 14. Mutants F61W, F124W, and F130W emerged later, showing maximum levels of RT activity at days 16–19. The RT activity detected in culture supernatants of mutants F124W and F130W was also very low, representing less than 20% of that shown by cultures transfected with the WT provirus. Viruses recovered from those cultures transfected with mutants F124W and F130W were viable, and their RT-coding sequence was identical to the one present in the proviral DNA. Reversions or additional mutations in the RT-coding region were not found in the progeny virus in any of the described transfection experiments. Virus titers of mutants F124W and F130W were usually around 10^4 50% tissue culture infective doses ($TCID_{50}$) per ml, while the other viable mutants showed titers similar to the WT virus, and in the range of 10^5 to 10^6 $TCID_{50}$ per ml.

Cultures transfected with mutants F77W and Y146W failed to produce any detectable RT activity even after 50 days of culture (values indistinguishable from those obtained in mock-transfection assays), were negative for the presence of p24, and supernatants from those cultures were unable to infect MT-4 cells.

The replication capacity of all viable mutants was determined upon infection of MT-4 cells by monitoring RT

Table 1
Maximum levels of RT activity in culture supernatants from cells transfected with HIV-1 cDNA clones carrying mutations in the RT-coding region[a]

Mutation	Day	RT activity (%)
WT	12	100
Y56W	12	90
F61W	16	60
F77W	n.d.[b]	n.d.
F87W	12	85
F116W	14	100
F124W	16	14
Y127W	12	90
F130W	19	17
Y144W	12	90
Y146W	n.d.	n.d.
F171W	12	101
Y181W	14	70
Y183W	12	70
Y188W	10	65
F227W	12	125
Y232W	12	89

[a] RT activity was monitored periodically in the cell culture medium of all transfections. Reported data refer to the day after transfection when the peak of RT activity was observed. RT activity values are referred to those obtained with cultures transfected with WT HIV-1. One microliter of culture supernatant produced 10,000 photo-stimulated light emission (PSL) units.
[b] n.d. stands for not detected. RT activity levels were not distinguishable from background (mock-infected cells) at all times tested (up to 50 days after transfection). Same results were obtained in at least three independent experiments.

activity and p24 antigen levels. Most viruses showed similar replication kinetics in comparison with WT HIV-1, with peaks of RT activity between days 7 and 9, and RT activity and p24 antigen levels above 60% of the WT virus (Fig. 1). However, mutants F124W and F130W showed impaired replication capacity with RT activity levels below 20% of the WT virus, and reduced amounts of p24 in culture supernatants.

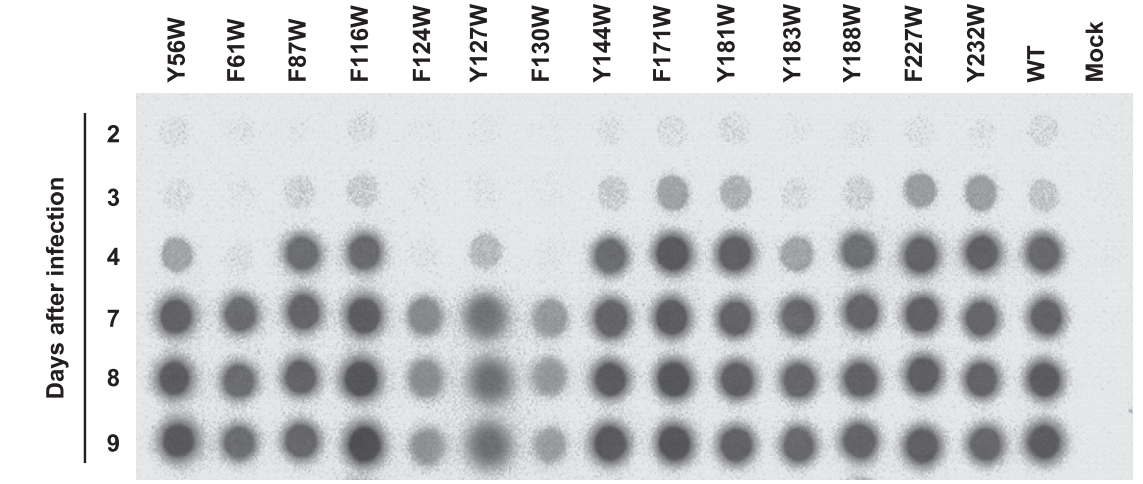
Enzymatic characterization of RT mutants

The RT polymerase activity conferred by mutations showing the largest effects on viral replication capacity (e.g., F61W, F77L, F124W, F130W and Y146W) was determined with the corresponding recombinant RTs. F116W was also included in this analysis as an example of a mutation producing a minor effect on viral replication. Expression and purification of all mutant RTs were rather efficient, except in the case of F130W. No protein was recovered when the mutation F130W was present in p51, as found with heterodimers $p66^{F130W}/p51^{F130W}$ (designated as F130W) or $p66^{WT}/p51^{F130W}$, suggesting that Phe-130 could be important to maintain the stability of p51. After induction with isopropyl-β-D-thiogalactopyranoside, the p51 subunit carrying the mutation F130W was expressed at levels below 10% of those observed with the WT polypeptide and was found to be very sensitive to proteolytic degradation during the purification process (data not shown). On the other hand, the heterodimeric RT containing the mutation F130W in p66, but having a WT p51 ($p66^{F130W}/p51^{WT}$), was purified in significant amounts and had a DNA-dependent DNA polymerase activity similar to that shown by the WT enzyme (Fig. 2). Qualitative assessments of the DNA polymerase activity of the mutant RTs, based on primer extension assays, indicated that Y146W and particularly F77W have a strongly impaired polymerase activity (Fig. 2).

Steady-state kinetic analysis of nucleotide incorporation by WT and mutant RTs revealed large differences between the WT enzyme and mutants F77W and Y146W (Table 2). The catalytic efficiencies of those mutants were reduced by 50-fold in comparison with WT RT due to their relatively high K_m values. Reduced catalytic efficiency appears as the likely cause of the replication defect observed in viruses containing mutations F77W or Y146W. Other tested mutations had a small effect on DNA polymerase activity, and their catalytic efficiencies were within a 2.5-fold range compared with the WT RT.

Analysis of compensatory mutations arising after passaging replication-deficient virus in cell culture

Viral replication assays showed that mutations F124W and F130W were the ones that produced the largest effect while maintaining virus viability. In an attempt to improve viral fitness, HIV-1 (strain 89ES061) bearing mutations F124W or F130W was passaged in MT-2 cells. In general, a new passage was done when cell viability decreased to less than 40%. At first, passages were carried out every 8–10 days, but cell viability decreased more rapidly in successive passages. At passage 8 with F124W or passage 6



	Y56W	F61W	F87W	F116W	F124W	Y127W	F130W	Y144W	F171W	Y181W	Y183W	Y188W	F227W	Y232W	WT	Mock
Relative RT activity (%)	98	55	75	130	20	56	13	100	120	90	68	80	97	75	100	<1
Relative p24 level (%)	91	65	115	102	38	110	43	92	89	96	89	101	100	77	100	<10

Fig. 1. Replication kinetics of WT and mutant HIV-1 in MT-4 cells. MT-4 cells (10^6) were infected with 0.0001 $TCDI_{50}$ per cell with viral stocks of the corresponding mutants. Samples were withdrawn from the infected cultures at the indicated times and virion-associated RT activity was determined using poly(rA)/oligo$(dT)_{12-18}$ and [^{32}P]dTTP as substrates. RT activity and p24 antigen levels at day 8 are given as percentages of the measurements obtained with the WT virus. Interassay variability was below 15%.

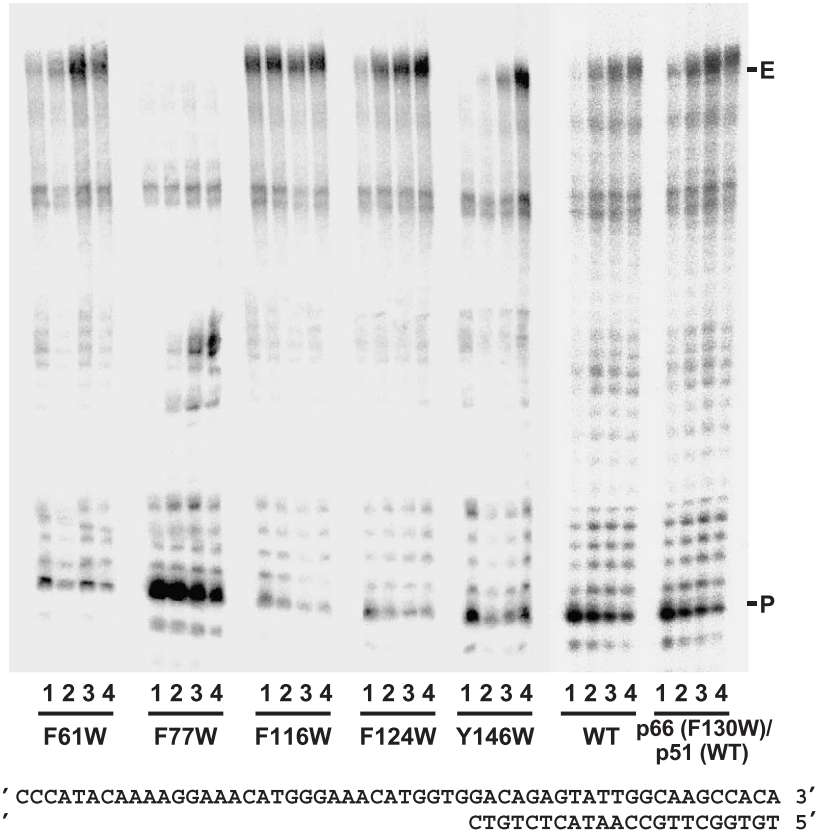


Fig. 2. Extension of primer 3TRP by WT and mutant RTs in assays containing a DNA template (M_54) and a mixture of dNTPs at a concentration of 0.5 mM each. Template-primer concentration was 30 nM, and reactions were incubated for 5, 15, 30, and 60 min (lanes 1–4) in the presence of a 12-nM concentration of each enzyme. P and E indicate the positions of the primer and the fully extended product, respectively. The sequence of the template-primer (M_54/3TRP) is given below.

with F130W, more than 40% of the cells were dead after 3–4 days. Nucleotide sequencing of the *gag* and *pol* genes of virus recovered at passage 8 showed that for mutant F124W, there was only one additional mutation, which was located at position 83 of the RT (K83R). In the case of mutant F130W, we found that the RT-coding region of virus recovered at passage 6 contained only one additional amino acid substitution (T58S) in comparison with the RT of the initial virus population, while no other mutations were found in the *gag* and *pol* genes.

Table 2
Kinetic parameters for dNTP incorporation of WT and mutant RTs[a]

Enzyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
WT	2.77 ± 1.28	0.32 ± 0.10	8.66 ± 2.27
F61W	1.50 ± 0.29	0.44 ± 0.19	3.39 ± 0.02 (0.4)[b]
F77W	2.39 ± 0.28	>100	<0.02 (<0.005)
F116W	1.00 ± 0.24	0.073 ± 0.046	13.7 ± 4.9 (1.6)
F124W	1.00 ± 0.18	0.051 ± 0.027	19.6 ± 10.3 (2.3)
$p66^{F130W}/p51^{WT}$	2.75 ± 0.46	0.38 ± 0.07	7.57 ± 2.68 (0.9)
Y146W	1.98 ± 0.79	12.2 ± 5.0	0.18 ± 0.11 (0.02)

[a] The template-primer used in these assays (D2–47/PG5–25) was 5′-GGGATTAAATAAAATAGTAAGAATGTATAGCCCTACCAG-CATTCTGG-3′ and 3′-TACATATCGGGATGGTCGTAAGACC-5′. Elongation reactions were incubated for 0–90 s for the incorporation of T. Data shown are the mean values ± standard deviations obtained from a nonlinear least square fit of the kinetics data to the Michaelis-Menten equation. Each of the experiments was performed independently at least twice. Mutations were introduced in both subunits of the heterodimer, except in the case of $p66^{F130W}/p51^{WT}$. Superscripts are used to indicate the amino acid substitutions found in each subunit.

[b] Values between parenthesis represent the ratio of the catalytic efficiency of each mutant enzyme relative to that shown by the WT RT.

Stocks of virus recovered from passage 6 or 8 in both experiments were obtained, titrated, and used to infect MT-4 cells. The growth curves of WT HIV-1 and mutants F124W, F130W, K83R/F124W, and T58S/F130W are shown in Fig. 3. For the WT virus, maximum RT activity was detected 7 days after infection, while cell viability decreased below 20%. Interestingly, the double-mutant K83R/F124W showed increased RT activity in viral supernatants and higher cytopathicity in comparison with the single-mutant F124W (Fig. 3A). However, the double-mutant K83R/F124W was still less virulent than the WT virus. On the other hand, T58S has a strong compensatory effect on the replication of mutant HIV-1 harboring mutation F130W (Fig. 3B). The RT activity in the supernatants of cultures infected with virus containing mutations T58S and F130W increased by more than 20-fold at day 7 compared with the single-mutant F130W. In addition, the cytopathic effect

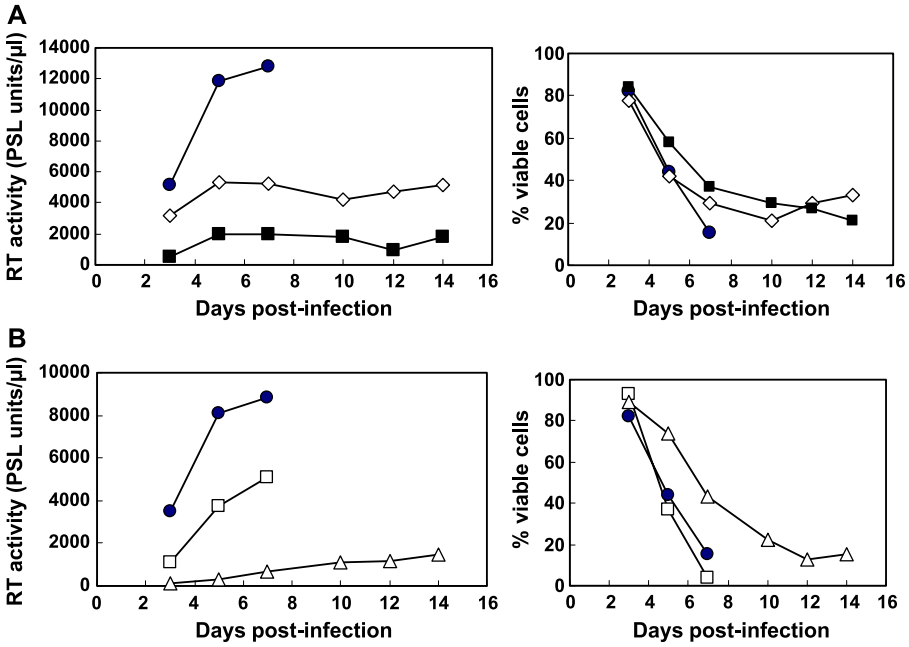


Fig. 3. Replication kinetics of WT HIV-1, single mutants F124W and F130W, and double mutants K83R/F124W and T58S/F130W. MT-4 cells (10^6) were infected at a multiplicity of infection of 0.0001 $TCDI_{50}$ per cell. Infections were monitored by measuring RT activity (left) and cell viability (right). (A) Comparison of WT HIV-1 (filled circles) with mutants F124W (filled squares) and K83R/F124W (open diamonds). (B) Comparison of WT HIV-1 with mutants F130W (open triangles) and T58S/F130W (open squares).

produced by the double-mutant T58S/F130W was similar to that observed with the WT virus.

Western blot analysis of the time course of infection with WT HIV-1 and mutants F130W and T58S/F130W was carried out with antibodies recognizing the viral capsid protein p24 (Fig. 4). In agreement with results described above, virus production was delayed in the case of mutant F130W (Fig. 4B) in comparison with the WT virus and the double-mutant T58S/F130W (Figs. 4A and C). Large amounts of p24 and the 55-kDa Gag precursor were observed in cells infected with all three viruses. However, a large amount of mature p24 relative to its Gag precursor was found only in virions from cell cultures infected with WT HIV-1 and mutant T58S/F130W. In the case of mutant F130W, the presence of significant amounts of unprocessed Gag in viral particles is consistent with an inefficient processing of the polyprotein precursor that delays virus maturation.

Further evidence of the compensatory effect of T58S was obtained from kinetic studies using recombinant RTs. Mutants T58S ($p66^{T58S}/p51^{T58S}$), T58S/F130W ($p66^{T58S/F130W}/p51^{T58S/F130W}$), and $p66^{WT}/p51^{T58S/F130W}$ showed high catalytic efficiency for nucleotide incorporation in DNA polymerase assays (Table 4). According to these data, the compensatory role of T58S can be attributed to the presence of both amino acid substitutions (T58S and F130W) in the 51-kDa subunit of HIV-1 RT, as demonstrated by using chimeric heterodimers where substitutions were introduced in p66, p51, or both subunits (Tables 2 and 3).

Discussion

The HIV-1 RT contains nine Phe and nine Tyr residues within the conserved motifs of DNA polymerases (Xiong and Eickbush, 1990). All of these residues are highly conserved among HIV-1 isolates from untreated individuals (Rhee et al., 2003). Frequent resistance mutations involving Tyr or Phe residues within the RT polymerase domain are F77L, F116Y, Y181C, Y188L, and F227L (reviewed in Menéndez-Arias, 2002b). Analysis of the crystal structure of the enzyme (Ding et al., 1998; Huang et al., 1998; Kohlstaedt et al., 1992) has shown that the side-chains of five residues within p66 (Phe-61, Tyr-115, Tyr-127, Tyr-181 and Tyr-183) and six residues within p51 (Phe-87, Phe-116, Tyr-127, Tyr-183, Phe-227 and Trp-229) are exposed to the solvent (e.g., residues with solvent-accessible surfaces below 10% of the maximum, as determined for the corresponding amino acid in an extended conformation).

Phe-61, Tyr-115, and Tyr-183 of the 66-kDa subunit are involved in interactions with the template primer or the incoming dNTP. Phe-61 contacts the first and second bases of the 5′-template overhang, the side-chain of Tyr-115 interacts with the ribose moiety of the nucleotide substrate, and Tyr-183 contacts the primer strand, at its 3′ end (Huang et al., 1998). Previous studies showed that recombinant RTs bearing mutations Y56W, F61W, Y181W, or Y232W retained high levels of DNA polymerase activity (Boyer et al., 1992; Fisher and Prasad, 2002; Ghosh et al., 1997; Sardana et al., 1992), while F87W has been found in one clinical isolate (Beerenwinkel et al., 2002). Nucleotide

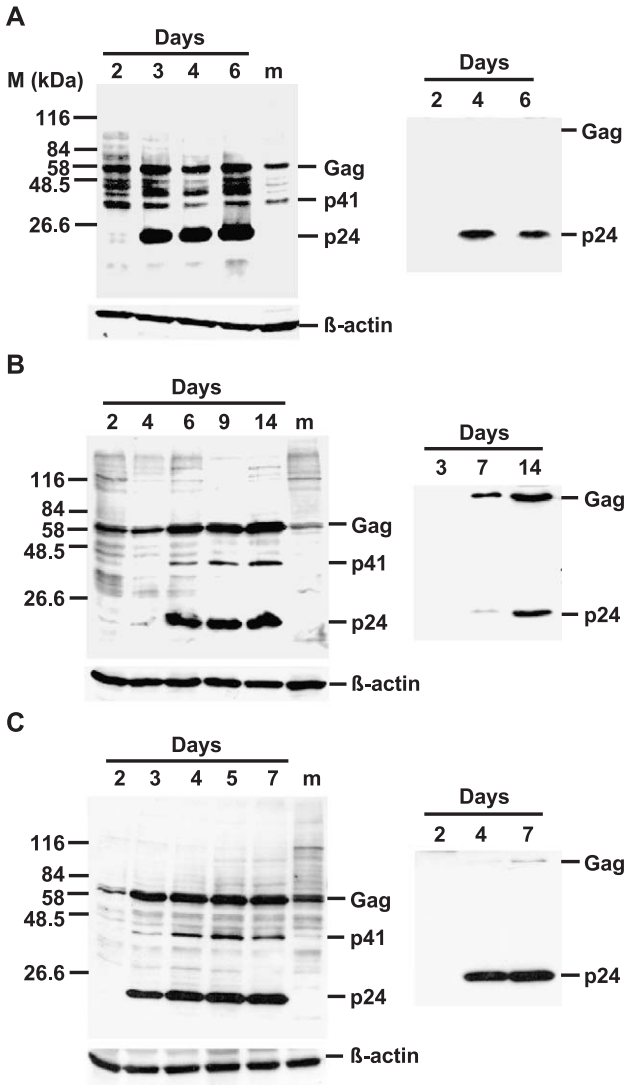


Fig. 4. Western blot detection of HIV-1 p24 in MT-4 cells infected with WT virus and mutants F130W and T58S/F130W. MT-4 cells were infected with 0.0001 $TDCI_{50}$ per cell and aliquots of culture supernatants and cells were collected daily until cell viability decreased to less than 10%. Infections were monitored by measuring RT activity, and representative aliquots were subjected to immunoblotting. The pellets obtained after processing 10^5 cells or 100 μl of culture supernatant were applied in SDS-polyacrylamide gels, and after electrotransfer, blots were developed with specific mouse monoclonal antibodies against p24. Western blots show the Gag processing time course of cultures infected with WT HIV-1 (A), mutant F130W (B), and the double-mutant T58S/F130W (C). Blots shown on the left were obtained with cell lysates, while those on the right were derived from the analysis of supernatant virions. A loading control for the cell lysate, obtained using a β-actin-specific antibody, is shown below each panel. m stands for mock-infected cells. Prestained protein molecular mass markers from Sigma were used as references. The electrophoretic mobilities of p24 and the 41- and 55-kDa precursors of p24 (p41 and Gag, respectively) are also indicated.

incorporation kinetics for mutant F61W and WT HIV-1 RT were rather similar (Fisher and Prasad, 2002), in agreement with data reported in this paper. However, Fisher et al. (2003) have shown that mutation F61W has a negative effect on the enzyme's ability to catalyze strand displacement synthesis on nicked and gapped duplex DNA templates. This alteration could explain the small reduction in viral replication capacity observed in our assays, with the mutant virus in comparison with the WT HIV-1. Our data indicate that most Phe and Tyr residues within the polymerase domain of HIV-1 RT can be replaced by Trp without major alterations of DNA polymerase function and virus viability.

In addition to Tyr-115 and Phe-160 of HIV-1 RT, whose replacement by Trp impairs dNTP binding during DNA polymerization, and have a deleterious effect on viral replication (Cases-González et al., 2000; Gutiérrez-Rivas et al., 1999; Martín-Hernández et al., 1996; Olivares et al., 1999), we found that substituting Trp for Phe-77 or Tyr-146 rendered nonviable virus, while mutants having Trp instead of Phe at positions 124 or 130 had a strongly diminished replication capacity. DNA polymerization assays showed that RTs carrying mutations F77W or Y146W had a very low catalytic efficiency for nucleotide incorporation due to their high apparent K_m values. Phe-77 is in the fingers subdomain and contacts Gln-151, whose side-chain interacts with the incoming nucleotide. The substitution of Leu for Phe-77 is usually associated with Q151M, and is commonly found in clinical isolates from patients treated with multiple nucleoside analogues (Shirasaka et al., 1995). This amino acid change leads to a side-chain volume reduction that does not affect the RT's DNA polymerase activity. In contrast, the substitution of Trp for Phe leads to a 29.2% increase of the side-chain volume that probably alters the conformation of the dNTP binding site in HIV-1 RT.

A similar mechanism could be invoked to explain the deleterious effect of Y146W. Tyr-146 occupies an internal position in both RT subunits, with less than 2% of its side-chain exposed to the solvent. Amino acids near Tyr-146 are Lys-46, Lys-73, Val-75, Arg-125, Thr-128, Phe-130, Gln-145, and Val-148, as well as dNTP-binding-site residues such as Phe-116 and Gln-151. Amino acid substitutions at position 146 are rare among HIV-1 clinical isolates (Rhee et al., 2003), and the Y146W mutation has not been

Table 3
Kinetic parameters for dNTP incorporation of RTs containing the amino acid substitution T58S[a]

Enzyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
T58S	0.71 ± 0.10	0.097 ± 0.012	7.29 ± 0.08
T58S/F130W	0.78 ± 0.06	0.183 ± 0.039	4.34 ± 0.90
$p66^{WT}/p51^{T58S/F130W}$	0.98 ± 0.16	0.155 ± 0.024	6.33 ± 0.62

[a] The template-primer used in these assays (D2–47/PG5–25) is given in Table 2. Elongation reactions were incubated for 0–90 s for the incorporation of T. Data shown are the mean values ± standard deviations obtained from a nonlinear least square fit of the kinetics data to the Michaelis-Menten equation. Each of the experiments was performed independently at least twice. Mutations were introduced in both subunits of the heterodimer, except in the case of $p66^{WT}/p51^{T58S/F130W}$. Superscripts are used to indicate the amino acid substitutions found in each subunit.

observed in HIV-infected patients. However, Trp has been found at the equivalent position of Tyr-146 of HIV-1 RT in other retroviral RTs, including those from murine leukemia virus, equine infectious anemia virus, human T cell leukemia virus types I and II, and mouse mammary tumor virus, among others (Xiong and Eickbush, 1990). Our data show that the recombinant RT bearing mutation Y146W has an impaired dNTP binding ability. This is probably due to an alteration of the conformation around the dNTP binding site in the 66-kDa subunit of the polymerase as a consequence of the packaging defect caused by the substitution of Trp for Tyr-146.

Compensatory mutations play a key role in virus adaptation. Thus, evolution of drug resistance is characterized by small to severe fitness losses that can be partially overcome by compensatory mutations or other adaptative changes that restore viral replication capacity (for a recent review, see Menéndez-Arias et al., 2003). Amino acid substitutions affecting aromatic residues within the RT polymerase domain that produce a drastic loss of viral replication capacity (e.g., Y115W or W229Y) can be compensated through the acquisition of second-site reversions that improve viral fitness. Examples are M230I for Y115W (Olivares et al., 1999) or the combination of I63M, V189I, and E369G for W229Y (Pelemans et al., 2001). In the first case, the double-mutant (Y115W/M230I) displayed WT replication capacity and emerged around 30–45 days after transfection, using the methodology described in this paper.

Emergence of second-site revertants was not observed at the end of transfection experiments with any of the mutants tested in this work. However, compensatory mutations were selected after passaging in cell culture the replication-deficient mutants F124W and F130W. The RT mutation K83R produced a partial but significant increase of the viral replication capacity of mutant virus harboring mutation F124W. The large impact of this mutation on viral replication capacity is difficult to explain from a structural point of view, because this residue is buried in the RT molecule and does not appear to be involved in functionally important interactions, such as template-primer binding, dNTP incorporation, or stabilization of the RT heterodimer. Besides, characterization of recombinant HIV-1 RT containing Trp at position 124 showed that this enzyme displayed DNA polymerase activity and nucleotide incorporation efficiency similar to those observed with the WT virus. Nevertheless, it should be noted that the RT of HIV-$1_{89ES061}$ contains Lys at position 83, instead of Arg as found in the recombinant RT used in our studies, that derives from HIV-1 strain BH10.

Phe-130 and Tyr-183 are the only aromatic amino acid residues of the HIV-1 RT polymerase domain that are strictly conserved in all retroviral RTs. The side-chain of Phe-130 is buried within the protein core in both RT subunits. RT heterodimers having the F130W substitution in p66 showed WT DNA polymerase activity. However, repeated attempts to express and purify RTs containing this mutation in p51 failed as a consequence of the lower stability of the 51-kDa subunit. The reasons of this stability loss are uncertain, although improper folding of p51, impaired RT heterodimerization, or higher susceptibility to proteases appear as likely causes of this defect. Interestingly, successive passages of the virus in cell culture led to selection of T58S as a second-site mutation that restored viral replication capacity in virus containing the RT mutation F130W.

Analyses of HIV-1 RT crystal structures indicate that Thr-58 interacts with Phe-130 (Fig. 5A). Thus, the closest distances between both residues in the ternary complex composed of HIV-1 RT, a template-primer, and an incoming dNTP (Huang et al., 1998) are 3.12 and 3.57 Å, for p51 and p66, respectively, which correspond to the distance between the -CO- group of Thr-58 and the Cβ atom of Phe-130. Similar distances were obtained with the coordinates of unliganded HIV-1 RT (Esnouf et al., 1995) and with coordinates of the enzyme complexed with a double-stranded DNA/DNA template-primer (Ding et al., 1998). Thr-58 is located within motif 1 in the fingers subdomain and is conserved in most retroviral RTs, including HIV-1 and other lentiviral polymerases (Xiong and Eickbush, 1990). Biochemical evidence reveals that the compensatory effect of the T58S mutation is mediated by p51. Thus, RTs containing both amino acid substitutions in p51 (e.g., $p66^{T58S/F130W}$/$p51^{T58S/F130W}$ and $p66^{WT}$/$p51^{T58S/F130W}$) were successfully expressed and purified, and showed kinetic properties similar to those displayed with the WT recombinant enzyme. In addition to the structural role, residues near Phe-130 such as Asn-57 and Arg-143 contribute to interactions with Trp-88 of the 66-kDa subunit (Fig. 5B) and could affect RT heterodimerization (Menéndez-Arias et al., 2001).

Western blot analysis confirmed that viral replication of mutant F130W was delayed in comparison with the WT HIV-1 or the double-mutant T58S/F130W. Although virions from cells infected with all three viruses contained significant levels of p24, we found large differences in the relative amounts of unprocessed Gag found in viral particles. Thus, the Gag precursor polypeptide was almost undetectable in the WT and T58S/F130W virions, but was present in large amounts in viral particles containing the mutation F130W in their RT-coding region (Fig. 4). These results show that F130W has a deleterious effect on virus maturation. Inefficient folding of one of the RT subunits is likely to impair the Pol dimerization process required to activate the viral protease.

There are very few examples of mutations in p51 affecting RT function. Thus, residues 136–139 of the β7–β8 loop of p51 are required to obtain functionally active RT heterodimers (Pandey et al., 2001), and the substitution of Glu-138 by Lys on the p51 subunit has been shown to confer resistance to various non-nucleoside RT inhibitors (Jonckheere et al., 1994; reviewed in Menén-

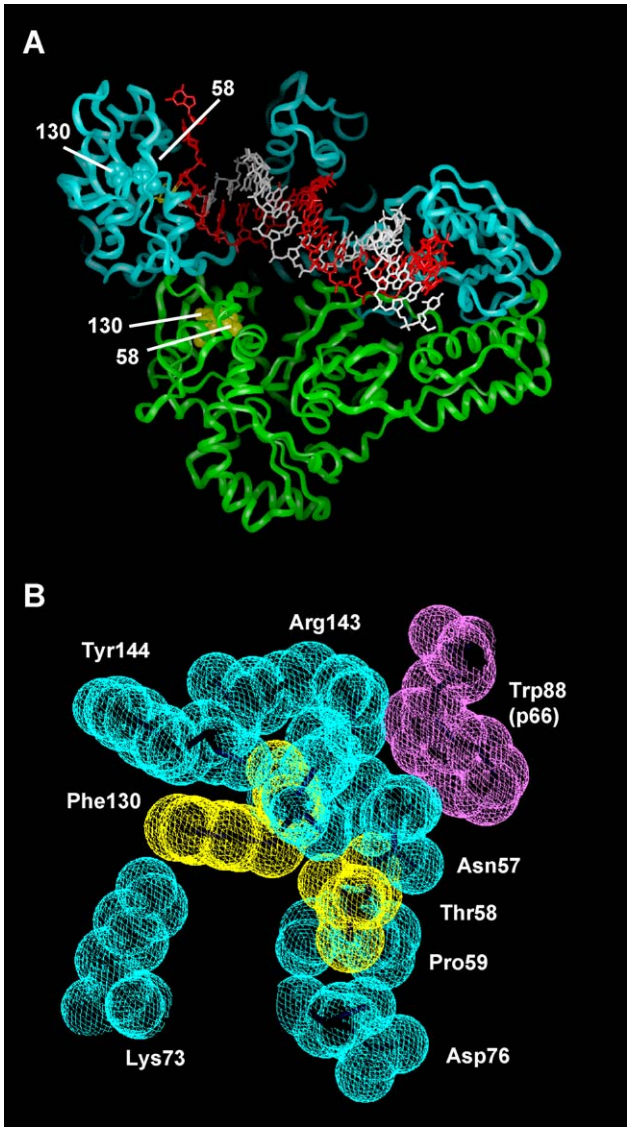


Fig. 5. Location of Thr-58 and Phe-130 in the crystallographic structure of HIV-1 RT. (A) Ribbon representation of the p66 (blue) and p51 (green) subunits of the RT complexed with a DNA/DNA template-primer (template shown in red, and primer in white) and dTTP, showing the location of residues 58 and 130 in both RT subunits. (B) Residues of p51 near Thr-58 and Phe-130. Van der Waals surfaces of these residues are shown in yellow, while those of amino acids within 3.5 Å of Thr-58 and Phe-130 are shown in blue (Asn-57, Thr-58, Pro-59, Lys-73, Asp-76, Ala-129, Thr-131, Arg-143, and Tyr-144). Trp-88 of p66 (magenta) interacts with p51 through residues Asn-57 and Arg-143.

dez-Arias, 2002b). However, our study is the first report where the structural integrity of p51 affecting the activity of the heterodimer is clearly demonstrated with virological studies. Furthermore, our results provide the first example of a compensatory mutation acting through p51. These findings illustrate the enormous adaptative potential of HIV-1 and should help in designing new strategies to abrogate RT function through a better knowledge of the molecular determinants involved in the formation of RT heterodimers.

Materials and methods

Cell lines and molecular clones

COS-1 cells and MT-4 cells were maintained as monolayer and suspension cultures, respectively, in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine. Virus used in this study was an infectious molecular clone of HIV-1 isolate 89ES061 previously described (Olivares et al., 1997, 1998). The 5′ end of the proviral DNA, including the 5′ long-terminal-repeat (LTR) region and the viral genes *gag*, *pol*, and *vif*, was cloned in the *Xba*I and *Eco*RI sites of plasmid pBSK (Stratagene) to obtain plasmid p61F A. The 3′ end of the proviral DNA, including the *env* gene and the 3′ LTR, was cloned in pBSK at the *Eco*RI and *Xba*I sites to generate plasmid p61F B. Co-transfection with plasmids p61F A and p61F B renders infectious virus after in vivo ligation (Olivares et al., 1997). Mutant HIV clones were obtained by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) by following the manufacturer's instructions. Mutations were introduced in plasmid p61F A using the primers shown in Table 4. Mutations were verified by DNA sequencing of the RT-coding region.

DNA transfection experiments

Transfections were performed as previously reported (Olivares et al., 1997). Briefly, 3×10^6 COS-1 cells were electroporated with 10 μg of subgenomic clones p61F A and p61F B. Forty-eight hours after transfection, 4×10^6 MT-4 cells were added to the culture. Viral replication was monitored by RT activity and p24 antigen detection in culture supernatants. Virion-associated RT activity was determined as described by Willey et al. (1988), after mixing 10 μl of transfection supernatant with 50 μl of an RT reaction mixture that contained a template-primer of poly(rA) (5 μg ml^{-1}) and oligo$(dT)_{12-18}$ (1.57 μg ml^{-1}) in 50 mM Tris, pH 7.8, 75 mM KCl, 2 mM dithiothreitol, 5 mM $MgCl_2$, 0.05% Nonidet P-40, and 0.5 μCi of [^{32}P]dTTP (800 Ci $mmol^{-1}$). Incorporated dTTP was estimated by phosphorimaging with a Fujifilm FLA-3000 scanner and the TINA 2.0 program. Production of p24 antigen in cell-free supernatants was measured using an antigen capture kit (SAIC Frederick, AIDS Vaccine Program).

Infections

To obtain a virus stock, supernatants from transfection experiments collected at days when maximum levels of RT activity were detected were used to infect 5×10^6 fresh MT-4 cells. Viral stocks were titrated in MT-2 cells and titers were calculated by the Spearman-Karber method (Mascola, 1999), and expressed as $TCID_{50}$. Infections were then carried out at a multiplicity of infection of 10^{-2} to 10^{-5} $TCID_{50}$ per cell. Viral replication was monitored by mea-

Table 4
Synthetic oligonucleotides used in site-directed mutagenesis reactions

Template	Mutation	Primers
p61F A	Y56W	5′-CGGGCCTGAAAATCCATGGAATACTCCAGTATTTGCC-3′
		5′-GGCAAATACTGGAGTATTCCATGGATTTTCAGGCCCG-3′
	F61W	5′-CGGGCCTGAAAATCCATATAATACTCCAGTATGGGCCATAAAG-3′
		5′-CTTTATGGCCCATACTGGAGTATTATATGGATTTTCAGGCCCG-3′
	F77W	5′-GTAGATTGGAGAGAACTTAATAAGAAAACTCAAGACTTCTGGG-3′
		5′-CCCAGAAGTCTTGAGTTTTCTTATTAAGTTCTCTCCAATCTAC-3′
	F87W	5′-GAAAACTCAAGACTGGTGGGAAGTTCAATTAGGAATACCACATCCC-3′
		5′-GGGATGTGGTATTCCTAATTGAACTTCCCACCAGTCTTGAGTTTTC-3′
	F116W	5′-GGGTGATGCATATTGGTCAGTTCCCTTAGATAAAGAATTCAGG-3′
		5′-CCTGAATTCTTTATCTAAGGGAACTGACCAATATGCATCACCC-3′
	F124W	5′-CAGTTCCCTTAGATAAAGAATGGAGGAAGTACACTGC-3′
		5′-GCAGTGTACTTCCTCCATTCTTTATCTAAGGGAACTG-3′
	Y127W	5′-CCCTTAGATAAAGAGTTCAGGAAGTGGACTGCATTTACCATACC-3′
		5′-GGTATGGTAAATGCAGTCCACTTCCTGAACTCTTTATCTAAGGG-3′
	F130W	5′-GGAAGTACACTGCATGGACCATACCTAGTATAAACAATGAGACAC-3′
		5′-GTGTCTCATTGTTTATACTAGGTATGGTCCATGCAGTGTACTTCC-3′
	Y144W	5′-GACACCAGGGATTAGATGGCAGTACAATGTGCTTCC-3′
		5′-GGAAGCACATTGTACTGCCATCTAATCCCTGGTGTC-3′
	Y146W	5′-GGGATTAGATATCAGTGGAATGTGCTTCCACAGGG-3′
		5′-CCCTGTGGAAGCACATTCCACTGATATCTAATCCC-3′
	F171W	5′-GCATGACAAAAATCTTAGAGCCTTGGAGAAAACAAAATCCAGAC-3′
		5′-GTCTGGATTTTGTTTTCTCCAAGGCTCTAAGATTTTTGTCATGC-3′
	Y181W	5′-CCAGACATAGTTATCTGGCAATACATGGACGATTTGTATGTAGG-3′
		5′-CCTACATACAAATCGTCCATGTATTGCCAGATAACTATGTCTGG-3′
	Y183W	5′-CCAGACATAGTTATCTATCAATGGATGGACGATTTGTATGTAGG-3′
		5′-CCTACATACAAATCGTCCATCCATTGATAGATAACTATGTCTGG-3′
	Y188W	5′-CAATACATGGACGATTTGTGGGTAGGATCTGACTTAGAAATAGGG-3′
		5′-CCCTATTTCTAAGTCAGATCCTACCCACAAATCGTCCATGTATTG-3′
	F227W	5′-CATCAGAAAGAACCTCCATGGCTTTGGATGGGTTATGAACTCC-3′
		5′-GGAGTTCATAACCCATCCAAAGCCATGGAGGTTCTTTCTGATG-3′
	Y232W	5′-CCTTTGGATGGGTTGGGAACTCCATCCTGATAAATGG-3′
		5′-CCATTTATCAGGATGGAGTTCCCAACCCATCCAAAGG-3′
pRT6	T58S	5′-GAGAATCCATACAATTCTCCAGTATTTGCC-3′
		5′-GGCAAATACTGGAGAATTGTATGGATTCTC-3′
	F61W	5′-CCATACAATACTCCAGTATGGGCCATAAAGAAAAAA-3′
		5′-TTTTTTCTTTATGGCCCATACTGGAGTATTGTATGG-3′
	F77W	5′-GGAGAAAATTAGTAGATTGGAGAGAACTTAATAAG-3′
		5′-CTTATTAAGTTCTCTCCAATCTACTAATTTTCTCC-3′
	F116W	5′-GTGGGTGATGCATATTGGTCAGTTCCCTTAGATG-3′
		5′-CATCTAAGGGAACTGACCAATATGCATCACCCAC-3′
	F124W	5′-GTTCCCTTAGATGAAGACTGGAGGAAGTATACTGC-3′
		5′-GCAGTATACTTCCTCCAGTCTTCATCTAAGGGAAC-3′
	F130W	5′-CAGGAAGTATACTGCATGGACCATACCTAGTATA-3′
		5′-TATACTAGGTATGGTCCATGCAGTATACTTCCTG-3′

suring RT activity in culture supernatants and estimating cell viability using the trypan blue staining method.

RNA isolation, amplification, and nucleotide sequence analysis

Culture supernatants were passed through 0.45-μl pore size filters and treated with DNase A for 30 min to eliminate input DNA. Total RNA was obtained from 20 μl of treated transfection supernatants (Boom et al., 1990). RNA amplification was done with the one tube RT-polymerase chain reaction (PCR) System (Titan, Boehringer Mannheim) using primers 47RU (5′ GTATTAGTAGGACCTACACCT 3′, positions 2055–2075) and 59RD (5′ ATGATTCCTAATGCATATTGTGAGT 3′, complementary to positions 3622–3646). Primers are numbered according to the sequence of Ratner et al. (1985). These primers amplify a 1591-base pair fragment. After reverse transcription at 50 °C for 30 min and a 5-min incubation at 94 °C, samples were subjected to 35 rounds of amplification. Each cycle composed a 1 min-denaturation step at 94 °C, a 1 min-annealing step at 55 °C, and a 2 min-extension step at 72 °C. Nucleotide sequence analysis of the amplified product was performed with primers 14RD (5′GCACGATATCTAATCCTGGTGTCTCA 3′, complementary to positions 2540–2561), 3′RU (5′ GCGGGATCCTGAAAATCCATACAATACTC 3′, positions 2278–2304), 15′RU (5′ TAGATATCAGTACAATGTGCTTCCAC 3′, positions 2555–2580), 58RU (5′ GCCA-

GAAAAAGACAGCTGGACTGT 3′, positions 2867–2890), and 59RD (5′ATGATTCCTAATGCATATTGTGAGT 3′ complementary to positions 3622–3646) by using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI-PRISM 377 DNA sequencer.

Immunoblotting

Aliquots of 450 μl of cell culture supernatants previously subjected to filtration through a 0.2-μm-pore size filter (Millipore, Bedford, MA) were overlaid onto 300 μl of a 20% sucrose cushion and centrifuged at 25 000 × *g* for 2 h at 4 °C. Virus pellets or MT-4 cells collected at different times after infection were resuspended in 80 mM Tris buffer, pH 6.8, containing 2% sodium dodecyl sulfate (SDS) and 1% glycerol, and then subjected to electrophoresis in an SDS-polyacrylamide gel. Separated proteins were electrotransferred to a polyvinylidene difluoride-type membrane (Hybond-PVDF, Amersham), and probed with mouse monoclonal antibodies against p24 or β-actin. Detection of membrane-bound antibodies was done using anti-mouse IgG labeled with horseradish peroxidase, and the reaction was developed with enhanced chemiluminescence detection reagents and Hyperfilm-ECL (Amersham). The monoclonal antibodies to HIV-1 p24 and β-actin were obtained from IBT Immunological and Biochemical Testsystems GmbH (Reutlingen, Germany) and Sigma, respectively.

Mutagenesis, expression, and purification of recombinant RTs

Site-directed mutagenesis was carried out with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) using plasmid pRT6 (Quiñones-Mateu et al., 1997) as template and the mutagenic primers shown in Table 4. The RT-coding regions of pRT6 and p61F A have different nucleotide sequences. Therefore, primers used to introduce mutations in pRT6 were different from those used to mutagenize p61F A, except in the case of Y146W, which was introduced in both plasmids with the same set of primers. Amino acid sequence differences between the RTs encoded within plasmids pRT6 and p61F A are R83K, E122K, D123E, K172R, R211K, L214F, R277K, K281R, E297K, V435I, P468T, L469I, N471D, Y483L, L517I, N519S, A534T, A554T, and I559V. None of these substitutions has been shown to affect RT function. After mutagenesis, the RT-coding regions were sequenced, and inserts containing the appropriate mutations were cloned in the p51 expression vector pT51H by following previously described procedures (Martín-Hernández et al., 1996; Menéndez-Arias, 1998). Purification of WT and mutant RTs was carried out after independent expression of their subunits (Martín-Hernández et al., 1996; Olivares et al., 1999). All RTs were purified as p66/p51 heterodimers. The 51-kDa polypeptide was obtained with an extension of 14 amino acid residues at its N-terminal end, which includes six consecutive histidines to facilitate its purification by metal chelate affinity chromatography.

Primer extension assays

The template/primer M_54/3TRP (sequences given in Fig. 2) was used to determine DNA-dependent DNA polymerase activity of mutant RTs. 3TRP was labeled at its 5′ terminus with [γ-^{32}P]ATP with T4 polynucleotide kinase and then annealed to M_54 in 150 mM NaCl and 150 mM magnesium acetate as described (Martín-Hernández et al., 1997). Fifteen microliters of a solution containing 10–15 nM enzyme and 60 nM template/primer in 100 mM HEPES, 30 mM NaCl, 30 mM magnesium acetate, 130 mM KCH_3COO, 1 mM dithiothreitol, and 5% (w/v) polyethylene glycol 6000, pH 7.0, was incubated at 37 °C for 10 min. Primer extensions were initiated by adding 15 μl of a mixture containing the four dNTPs at a concentration of 1 mM each in 130 mM KCH_3COO, 1 mM dithiothreitol, and 5% (w/v) polyethylene glycol 6000. Reactions were incubated for 0–60 min. Aliquots of 5 μl were withdrawn at different times and reactions were then stopped by adding 5 μl of 10 mM EDTA loading buffer containing 90% formamide. DNA synthesis products were separated on a 20% polyacrylamide-urea gel and visualized by phosphorimaging with a BAS 1500 scanner (Fuji).

Single nucleotide extension assays

The template/primer D2-47/PG5-25 (sequences given in Table 2) was used. PG5–25 was 5′-end-labeled and annealed to D2–47 as described above for M_54/3TRP. Nucleotide incorporation assays were performed in 10 μl of 50 mM HEPES, pH 7.0, 15 mM NaCl, 15 mM magnesium acetate, 130 mM KCH_3COO, 1 mM dithiothreitol, and 5% polyethylene glycol 6000. The template-primer concentration was 30 nM and the active enzyme concentration was around 6 nM. Reactions were initiated by incubating the enzyme with the corresponding annealed template-primer in the absence of dNTP (10 min at 37 °C), and then followed by the addition of appropriate dNTPs at various concentrations. The rate of product formation was measured for 10–14 different concentrations of the correct dNTP (dTTP). Elongation reactions for the incorporation of dTTP were carried out at 37 °C for 10–90 s. Under these conditions, reactions were linear with respect to time and proportional to the enzyme concentration. After incubation, reactions were stopped by adding EDTA (5 mM final concentration), followed by heat denaturation. Products were resolved by electrophoresis in 20% polyacrylamide-urea gels and quantitated with a BAS 1500 scanner. Elongation measurements were fitted to the Michaelis-Menten equation, and the k_{cat} and K_m values were determined as previously described (Martín-Hernández et al., 1996).

Acknowledgments

We thank Virginia Álvarez Nunes for technical assistance. Work at the C.B.M.S.O. was supported by grants from Fondo de Investigación Sanitaria (FIS)-FEDER (01/0067-01) and Ministerio de Ciencia y Tecnología (BIO2003-01175), and by an institutional grant of Fundación Ramón Areces. Work at C.N.M. was supported by grant SAF 2002-00626. Support from FIS through the "Red Temática Cooperativa de Investigación en SIDA (Red G03/173)" is also acknowledged.

References

Arts, E.J., Ghosh, M., Jacques, P.S., Ehresmann, B., Le Grice, S.F.J., 1996. Restoration of $tRNA^{Lys,3}$-primed (−)-strand DNA synthesis to an HIV-1 reverse transcriptase mutant with extended tRNAs: implications for retroviral replication. J. Biol. Chem. 271, 9054–9061.

Beerenwinkel, N., Schmidt, B., Walter, H., Kaiser, R., Lengauer, T., Hoffmann, D., Korn, K., Selbig, J., 2002. Diversity and complexity of HIV-1 drug resistance: a bioinformatics approach to predicting phenotype from genotype. Proc. Natl. Acad. Sci. U.S.A. 99, 8271–8276.

Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheim-van Dillen, P.M.E., van der Noordaa, J., 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28, 495–503.

Boyer, P.L., Ferris, A.L., Hughes, S.H., 1992. Mutational analysis of the fingers domain of human immunodeficiency virus type 1 reverse transcriptase. J. Virol. 66, 7533–7537.

Cases-González, C.E., Gutiérrez-Rivas, M., Menéndez-Arias, L., 2000. Coupling ribose selection to fidelity of DNA synthesis—The role of Tyr-115 of human immunodeficiency virus type 1 reverse transcriptase. J. Biol. Chem. 275, 19759–19767.

Di Marzo Veronese, F., Copeland, T.D., DeVico, A.L., Rahman, R., Oroszlan, S., Gallo, R.C., Sarngadharan, M.G., 1986. Characterization of highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III/LAV. Science 231, 1289–1291.

Ding, J., Das, K., Hsiou, Y., Sarafianos, S.G., Clark Jr., A.D., Jacobo-Molina, A., Tantillo, C., Hughes, S.H., Arnold, E., 1998. Structural and functional implications of the polymerase active site region in a complex of HIV-1 RT with a double-stranded DNA template-primer and an antibody Fab fragment at 2.8 Å resolution. J. Mol. Biol. 284, 1095–1111.

Esnouf, R., Ren, J., Ross, C., Jones, Y., Stammers, D., Stuart, D., 1995. Mechanism of inhibition of HIV-1 reverse transcriptase by non-nucleoside inhibitors. Nat. Struct. Biol. 2, 303–308.

Fisher, T.S., Prasad, V.R., 2002. Substitutions of Phe61 located in the vicinity of template 5′-overhang influence polymerase fidelity and nucleoside analog sensitivity of HIV-1 reverse transcriptase. J. Biol. Chem. 277, 22345–22352.

Fisher, T.S., Darden, T., Prasad, V.R., 2003. Substitutions at Phe61 in the β3–β4 hairpin of HIV-1 reverse transcriptase reveal a role for the fingers subdomain in strand displacement DNA synthesis. J. Mol. Biol. 325, 443–459.

Ghosh, M., Williams, J., Powell, M.D., Levin, J.G., Le Grice, S.F.J., 1997. Mutating a conserved motif of the HIV-1 reverse transcriptase palm subdomain alters primer utilization. Biochemistry 36, 5758–5768.

Gutiérrez-Rivas, M., Ibáñez, A., Martínez, M.A., Domingo, E., Menéndez-Arias, L., 1999. Mutational analysis of Phe160 within the "palm" subdomain of human immunodeficiency virus type 1 reverse transcriptase. J. Mol. Biol. 290, 615–625.

Harris, D., Lee, R., Misra, H.S., Pandey, P.K., Pandey, V.N., 1998. The p51 subunit of human immunodeficiency virus type 1 reverse transcriptase is essential for loading the p66 subunit on the template primer. Biochemistry 37, 5903–5908.

Huang, H., Chopra, R., Verdine, G.L., Harrison, S.C., 1998. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. Science 282, 1669–1675.

Jonckheere, H., Taymans, J.-M., Balzarini, J., Velázquez, S., Camarasa, M.-J., Desmyter, J., De Clercq, E., Anné, J., 1994. Resistance of HIV-1 reverse transcriptase against [2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)] (TSAO) derivatives is determined by the mutation Glu^{138}→Lys on the p51 subunit. J. Biol. Chem. 269, 25255–25258.

Joyce, C.M., Steitz, T.A., 1995. Polymerase structures and function: variations on a theme? J. Bacteriol. 177, 6321–6329.

Kohlstaedt, L.A., Wang, J., Friedman, J.M., Rice, P.A., Steitz, T.A., 1992. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science 256, 1783–1790.

Martín-Hernández, A.M., Domingo, E., Menéndez-Arias, L., 1996. Human immunodeficiency virus type 1 reverse transcriptase: role of Tyr115 in deoxynucleotide binding and misinsertion fidelity of DNA synthesis. EMBO J. 15, 4434–4442.

Martín-Hernández, A.M., Gutiérrez-Rivas, M., Domingo, E., Menéndez-Arias, L., 1997. Mispair extension fidelity of human immunodeficiency virus type 1 reverse transcriptases with amino acid substitutions affecting Tyr115. Nucleic Acids Res. 25, 1383–1389.

Mascola, J.R., 1999. Neutralization of HIV-1 infection of human peripheral blood mononuclear cells (PBMC): antibody dilution method. In: Michael, N.L., Kim, J.H. (Eds.), HIV Protocols. Methods in Molecular Medicine, vol. 17. Humana Press, Totowa, NJ, pp. 317–322.

Menéndez-Arias, L., 1998. Studies on the effects of truncating α-helix E′ of p66 human immunodeficiency virus type 1 reverse transcriptase on template-primer binding and fidelity of DNA synthesis. Biochemistry 37, 16636–16644.

Menéndez-Arias, L., 2002a. Molecular basis of fidelity of DNA synthesis and nucleotide specificity of retroviral reverse transcriptases. Prog. Nucleic Acid Res. Mol. Biol. 71, 91–147.

Menéndez-Arias, L., 2002b. Targeting HIV: antiretroviral therapy and development of drug resistance. Trends Pharmacol. Sci. 23, 381–388.

Menéndez-Arias, L., Abraha, A., Quiñones-Mateu, M.E., Mas, A., Camarasa, M.-J., Arts, E.J., 2001. Functional characterization of chimeric reverse transcriptases with polypeptide subunits of highly divergent HIV-1 group M and O strains. J. Biol. Chem. 276, 27470–27479.

Menéndez-Arias, L., Martínez, M.A., Quiñones-Mateu, M.E., Martinez-Picado, J., 2003. Fitness variations and their impact on the evolution of antiretroviral drug resistance. Curr. Drug Targets—Infect. Disord. 3, 355–371.

Nakamura, T.M., Morin, G.B., Chapman, K.B., Weinrich, S.L., Andrews, W.H., Lingner, J., Harley, C.B., Cech, T.R., 1997. Telomerase catalytic subunit homologs from fission yeast and human. Science 277, 955–959.

Olivares, I., Shaw, G., López-Galíndez, C., 1997. Phenotypic switch in a Spanish HIV type 1 isolate on serial passage on MT-4 cells. AIDS Res. Hum. Retroviruses 13, 979–984.

Olivares, I., Casado Herrero, C., Iglesias-Ussel, M.D., Dietrich, U., López-Galíndez, C., 1998. Complete sequence of an infectious molecular clone derived from a Spanish HIV type I isolate. AIDS Res. Hum. Retroviruses 14, 1649–1651.

Olivares, I., Sánchez-Merino, V., Martínez, M.A., Domingo, E., López-Galíndez, C., Menéndez-Arias, L., 1999. Second-site reversion of a human immunodeficiency virus type 1 reverse transcriptase mutant that restores enzyme function and replication capacity. J. Virol. 73, 6293–6298.

O'Reilly, E.K., Kao, C.C., 1998. Analysis of RNA-dependent RNA polymerase structure and function as guided by known polymerase structures and computer predictions of secondary structure. Virology 252, 287–303.

Pandey, P.K., Kaushik, N., Talele, T.T., Yadav, P.N.S., Pandey, V.N., 2001. The β7-β8 loop of the p51 subunit in the heterodimeric (p66/p51) human immunodeficiency virus type 1 reverse transcriptase

is essential for the catalytic function of the p66 subunit. Biochemistry 40, 9505–9512.

Pelemans, H., Esnouf, R., Min, K.-L., Parniak, M., De Clercq, E., Balzarini, J., 2001. Mutations at amino acid positions 63, 189, and 396 of human immunodeficiency virus type 1 reverse transcriptase (RT) partially restore the DNA polymerase activity of a Trp229Tyr mutant RT. Virology 287, 143–150.

Quiñones-Mateu, M.E., Soriano, V., Domingo, E., Menéndez-Arias, L., 1997. Characterization of the reverse transcriptase of a human immunodeficiency virus type 1 group O isolate. Virology 236, 364–373.

Ratner, L., Haseltine, W., Patarca, R., Livak, K.J., Starcich, B., Josephs, S.F., Doran, E.R., Rafalski, J.A., Whitehorn, E., Baumeister, K., Ivanoff, L., Petteway Jr., L.R., Pearson, M.L., Lautenberger, J.A., Papas, T.S., Ghrayeb, J., Chang, N.T., Gallo, R.C., Wong-Staal, F., 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature 313, 277–284.

Rhee, S.-Y., Gonzales, M.J., Kantor, R., Betts, B.J., Ravela, J., Shafer, R.W., 2003. Human immunodeficiency virus reverse transcriptase and protease sequence database. Nucleic Acids Res. 31, 298–303.

Sardana, V.V., Emini, E.A., Gotlib, L., Graham, D.J., Lineberger, D.W., Long, W.J., Schlabach, A.J., Wolfgang, J.A., Condra, J.H., 1992. Functional analysis of HIV-1 reverse transcriptase amino acids involved in resistance to multiple nonnucleoside inhibitors. J. Biol. Chem. 267, 17526–17530.

Shirasaka, T., Kavlick, M.F., Ueno, T., Gao, W.-Y., Kojima, E., Alcaide, M.L., Chokekijchai, S., Roy, B.M., Arnold, E., Yarchoan, R., Mitsuya, H., 1995. Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. Proc. Natl. Acad. Sci. U.S.A. 92, 2398–2402.

Svarovskaia, E.S., Cheslock, S.R., Zhang, W.-H., Hu, W.-S., Pathak, V.K., 2003. Retroviral mutation rates and reverse transcriptase fidelity. Front. Biosci. 8, D117–D134.

Telesnitsky, A., Goff, S.P., 1997. Reverse transcriptase and the generation of retroviral DNA. In: Coffin, J.W., Hughes, S.H., Varmus, H.E. (Eds.), Retroviruses. Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 121–160.

Willey, R.L., Smith, D.H., Lasky, L.A., Theodore, T.S., Earl, P.L., Moss, D., Capon, D.J., Martin, M.A., 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. 62, 139–147.

Xiong, Y., Eickbush, T.H., 1990. Origin and evolution of retroelements based upon their reverse transcriptase sequences. EMBO J. 9, 3353–3362.